MycAssay™ Pneumocystis
Applied BioSystems 7500
Respiratory Samples
REF 080-035

Intended Use:

MycAssay™ Pneumocystis is indicated for use by qualified laboratory professionals for the qualitative detection of *Pneumocystis jirovecii* genomic DNA extracted from respiratory specimens from the lower respiratory tract (e.g., bronchial samples) as an aid to diagnosis in adult patients suspected of having *P. jirovecii* pneumonia.

MycAssay™ Pneumocystis has been validated for use with the Applied BioSystems 7500 (using SDS software version 1.4).

Summary and Explanation

Pneumocystis jirovecii (formerly *carinii*) pneumonia (PCP) is a common opportunistic pneumonia in immunocompromised patients, especially those with advanced HIV infection and AIDS[1]. It is typically community acquired, and sub-acute in presentation, leading to progressive respiratory failure and death[2] if untreated. Prophylaxis with trimethoprim-sulphamethoxazole (Bactrim or Septrin) is routinely given to many at risk patients, a practice which has substantially reduced the incidence of PCP, but breakthrough occurs and those who do not know they are HIV positive may present with

[1] Morris A, Lundgren JD, Masur H, Walzer PD, Hanson DL, Frederick T, Huang L, Beard CB, Kaplan JE. (2004). Current epidemiology of *Pneumocystis* pneumonia. Emerg Infect Dis: 10: 1713-20.
[2] Miller RF, Allen E, Copas A, Singer M, Edwards SG. Improved survival for HIV infected patients with severe *Pneumocystis jirovecii.* pneumonia is independent of highly active antiretroviral therapy. Thorax 2006; 61:716-21.

AIDS with PCP[3]. PCP also occurs in other immunocompromised patients, including recipients of solid organ transplants, hypogammaglobulinaemia and chronic leukaemia.

Currently the diagnosis of PCP relies on microscopic methods as *P. jirovecii* cannot be cultured in routine microbiology laboratories. Bronchoalveolar lavage (BAL) is the preferred means of sample collection. Common methods for diagnosis include immunofluorescence (IF) or direct fluorescence and histological staining of samples[4].

MycAssay™ Pneumocystis is a molecular diagnostic kit for the detection of *P. jirovecii* based on Molecular Beacon[5] PCR technology. The whole test procedure, including extraction of DNA from the clinical sample, can be completed within 4 hours, or only 2 hours if extracted DNA is already available. This assay brings the direct benefit of enhanced laboratory efficiency combined with a rapid test leading to likely clinical benefits. The diagnostic accuracy of the test depends to a great extent on sample quality.

Principles of the Procedure

Following mixing of the reagents in the MycAssay™ Pneumocystis kit with a sample containing *Pneumocystis* target DNA sequence, (a portion of the *Pneumocystis* mitochondrial ribosomal large sub-unit), thermocycling will result in DNA amplification occurring. The assay also contains an Internal Amplification Control (IAC) sequence, a DNA fragment not present in *Pneumocystis*, other fungal, bacterial or human genomes, to detect PCR inhibitory substances and confirm the functionality of the assay reagents.

The amplified DNA targets are detected with Molecular Beacons; single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure. The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore, which fluoresces when excited by light of the appropriate wavelength, is covalently linked to the end of one arm and a quencher, which suppresses the fluorescence of the fluorophore when in close physical proximity, is covalently linked to the end of the other arm. Molecular Beacons do not fluoresce when they are free in solution. However, when they hybridise to a nucleic acid strand containing a target sequence they undergo a conformational change that enables them

[3] Kovacs JA, Gill VJ, Meshnick S, Masur H. (2001). New insights into transmission, diagnosis, and drug treatment of *Pneumocystis carinii* pneumonia. JAMA: 286: 2450-60.
[4] Huang L, Morris A, Limper AH, Beck JM; ATS *Pneumocystis* Workshop Participants. An Official ATS Workshop Summary: Recent advances and future directions in pneumocystis pneumonia (PCP). Proc Am Thorac Soc 2006;3:655-64.
[5] Tyagi S, Kramer FR. (1996). Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology: 14: 303-308.

to fluoresce. The amount of fluorescence at any given cycle, or following cycling, depends on the amount of specific amplicons present at that time. The Real-Time PCR System simultaneously monitors the fluorescence emitted by each beacon.

Precautions

- The kit is intended for use only by laboratory professionals. Procedures are required for non-aerosol manipulations of specimens. Standard precautions and institutional guidelines should be followed in handling all samples. A Material Safety Data Sheet is available from Myconostica Ltd.
- This test is for *in vitro* diagnostic use only.
- This test is only for use with the Applied BioSystems 7500 with SDS software version 1.4.
- MicroAmp® Optical PCR tube strips (see Equipment/Materials section for more details) should be used with this kit. Use of different plastic consumables could invalidate the assay results.
- Do not use reagents or controls if the protective pouches are open or broken upon arrival.
- Reagents and controls are not interchangeable between kits with differing lot numbers.
- Never pool reagents or controls from different tubes even if they are from the same lot.
- Never use the reagents or controls after their expiry date.
- Reagents and controls should not be refrozen or reused after opening.
- Wear protective clothing and disposable gloves while handling kit reagents.
- Avoid microbial and deoxyribonuclease (DNase) contamination of reagents when removing aliquots from tubes.
- The use of sterile DNase-free, low-retention disposable filter-tipped or positive displacement pipette tips is recommended.
- Use a new tip for each specimen or reagent.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- To avoid contamination with *Pneumocystis* or internal amplification control (IAC) amplicons, do not open the reaction tubes post-amplification.
- Additional controls may be tested according to guidelines or requirements of local, state, provincial and/or federal regulations or accrediting organisations.
- Do not eat, drink or smoke in areas where specimens or kit reagents are being handled.
- Low concentrations of DNA can be unstable if not stored correctly. It is recommended that DNA extractions from clinical samples are stored at -80^{o}C to preserve their integrity. Multiple rounds of thawing and refreezing should also be avoided whenever possible.

Kit Contents

Description
The kit consists of five 3-compartment sealed foil pouches, each of which can be used separately. Each pouch contains sufficient reagents for 8 reactions.

		Volume
Tube 1 (Orange Cap)	dNTPs $MgCl_2$ Buffered solution of DNA Polymerase complex	66 µL
Tube 2 (Blue Cap)	<0.01% Primers <0.01% Molecular Beacons <0.0001% Internal Amplification Control (IAC) The Internal Amplification Control is a recombinant DNA plasmid harbouring a non-infective sequence unrelated to either target (*Pneumocystis*) sequence Tris-HCl Buffer	66 µL
Tube 3 (Clear Cap)	Negative Control Water	25 µL
Tube 4 (Black Cap)	Positive Control <0.0001% Positive Control DNA The Positive Control molecule is a recombinant plasmid harbouring the *Pneumocystis* target sequences Tris-HCl Buffer	25 µL

The kit also contains:
- MycAssay™ Pneumocystis Myconostica Protocol CD-ROM
- Instructions for Use
- Certificate of Analysis

Storage

The kit should be stored frozen (-15 to -25 °C) until the expiry date indicated on the kit box label, at which time it should be disposed of according to local regulations.

Once a pouch has been opened, the contents must be used immediately, not re-frozen or re-used.

Equipment/Materials required and not provided

- Applied BioSystems 7500 Real-Time PCR System (including user manual, attached computer and SDS software version 1.4).
- MicroAmp® Optical 8-tube strip (Applied BioSystems, part number:4316567).
- MicroAmp® Optical 8-cap strip (Applied BioSystems, part number:4323032).
- Micro centrifuge with 0.2 mL PCR tube adapter.
- Vortex mixer
- Support rack for PCR tubes.
- Micropipettes (volumes required 7.5 µL – 20 µL)
- Sterile low-retention filtertips
- Disposable gloves, powderless
- Proprietary DNA decontaminating solution
- Permanent marker pen
- DNA isolation kit (see below)

Specimen

The specimen for the MycAssay™ Pneumocystis assay is total DNA extracted from clinical BAL samples. The following DNA isolation kit and equipment, supplied by Myconostica Ltd., is recommended for this purpose and was used during validation:

- MycXtra® Fungal DNA Extraction kit (REF: 080-005 available from Myconostica)
- Vortex-Genie 2 (Scientific Industries Inc., New York, USA)
- Vortex Adapter Plate (REF: 080-015 available from Myconostica)

Procedural Notes

- Read the entire protocol before commencing
- The entire MycAssay™ Pneumocystis process (excluding DNA extraction) takes approximately 2 hours, dependent on the number of samples tested.
- Setting up of the test should be performed in a PCR workstation or pre-PCR laboratory. If a PCR workstation is not available, then the test should be set-up in a dedicated area of the laboratory[6], which is regularly cleaned with DNA decontaminating reagents.

[6] For example see Mifflin, T. E. (2003). Setting up a PCR Laboratory. *In* PCR Primer, 2nd Ed. (eds. Dieffenbach and Dveksler). Cold Spring Harbour Laboratory Press, Cold Spring Harbour, NY. USA.

- However, avoid using DNA decontaminating reagents during the Real-Time PCR set-up as they can inhibit the assay.
- Use micropipettes for the transfer of fluids. Dedicated micropipettes should be used for the set-up of these reactions and they should be regularly decontaminated.
- Low-retention filtertips are recommended for use to ensure that no DNA is lost during the set-up procedure.
- **Exercise caution when handling Tube 4. This contains template DNA material and contamination could result in false positive test results.**
- Wear gloves at all times.
- All tubes must be capped following use and prior to disposal.
- Accurately note the positions of samples when multiple patient samples are being processed.

Procedure for Use:

1. Real-Time PCR Set-Up

1.1 To begin, switch on the AB7500 Real-Time PCR System (instrument and associated computer) and launch the SDS v1.4 software. Enter usernames and passwords if required.

1.2 Ensure the work area has been cleaned using DNA decontaminating reagents and allowed to dry completely; avoid use during assay set-up as excess cleaning solution may inhibit the PCR reactions.

1.3 A pouch contains one each of Tube 1, Tube 2, Tube 3 and Tube 4. There are sufficient reagents in one pouch to run 8 reactions. At least one positive control and one negative control reaction must be performed per run where the reagents are from a single kit lot. One pouch therefore can analyse 6 patient samples. If more than 6 samples need to be tested, more than one pouch can be used if the pouches used are from the same kit lot. A maximum of 38 patient samples may be tested using the 5 pouches in a kit.

1.4 Calculate the number of reactions required, referring to the table below:

Number of Pouches	Maximum number of patient samples
1	6
2	14
3	22
4	30

5	38

1.5 Remove the appropriate number of pouches from the freezer. Do not use any pouch that is no longer sealed. If the patient samples were frozen after extraction, also remove these from the freezer.

1.6 Tear open the required number of pouches and remove the tubes. If more than one pouch is being used, but only one set of positive and negative controls are being run, it is only necessary to remove Tubes 3 and 4 from one pouch. **Exercise caution when handling Tube 4. This contains positive control DNA material and contamination could cause false positive test results.**

1.7 Allow the tubes' contents to thaw by placing on the laboratory bench for 5-10 minutes, ensuring that the contents of each tube are completely thawed before proceeding. Vortex to mix the tubes' contents and the patient samples; follow by a short spin in a microcentrifuge to ensure collection of all the contents at the base of the tubes before use.

1.8 Place the required number of PCR tubes in the support rack. **Never touch the bottom of the reaction tubes with your hands.**

1.9 Always set up the negative control first, followed by the patient samples. The positive control should always be set up last.

1.10 Reagent and DNA volumes are shown in the table below:

Reagent	Reaction		
	Negative control	**Patient sample**	**Positive control**
Tube 1 (Orange cap)	7.5 µL	7.5 µL	7.5 µL
Tube 2 (Blue cap)	7.5 µL	7.5 µL	7.5 µL
Tube 3 (Clear cap)	10 µL	-	-
Patient Sample	-	10 µL	-
Tube 4 (Black cap)	-	-	10 µL
Total volume	25 µL	25 µL	25 µL

1.11 Add reagents in the order shown in the table above; Tube 1, then Tube 2, followed by the template (Negative control, Patient sample, or Positive

control). Take care when taking aliquots from Tube 1; the liquid is slightly viscous and can stick on the inner ridge of the tube. If this happens, re-spin to collect the final contents in the base of the tube before attempting to remove the final aliquots.

1.12 Use a new pipette tip for every liquid transfer. Re-cap each reagent tube after use and immediately discard it, and any remaining contents, into a sealable clinical waste container. Unused reagents cannot be saved for later use.

1.13 Take extra care when pipetting Tube 4 (positive control DNA) to ensure it does not contaminate any other reaction tube. Closing the lids on the other reaction tubes before opening Tube 4 can reduce the risk of cross-contamination.

1.14 Make sure all reaction tube caps are firmly closed. Make a note of the positions of each sample in the tube strip. Label (for example: on the lid) the first tube of each strip if more than one strip is used. Spin down the reaction tubes for 10 seconds using a mini centrifuge with 0.2 mL PCR tube adapter. Visually check that there are no bubbles present in the reaction mixtures.

1.15 Proceed to Section 2 promptly. MycAssay™ Pneumocystis reactions are stable on the bench for up to 60 minutes.

1.16 Following the PCR set-up ensure the work area is thoroughly cleaned using DNA decontaminating reagents.

2. Performing the run

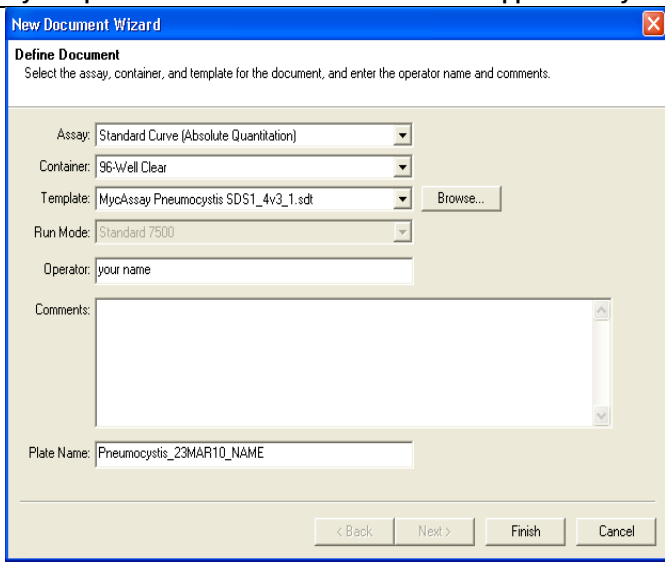
2.1 Open up the AB 7500 SDS software version 1.4 and enter your username and password.

2.2 Insert the **MycAssay Pneumocystis Myconostica Protocol CD-ROM**.

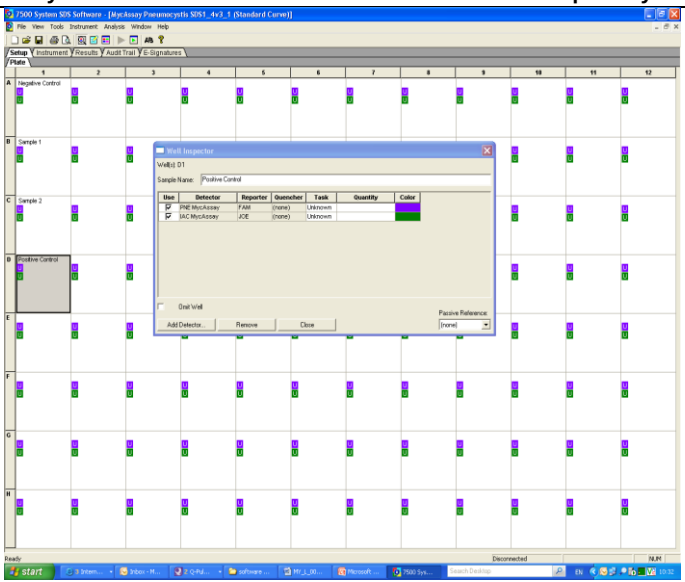
2.3 In the **Quick Startup** menu, select the first option; **Create New Document...**

2.4 Choose the settings as shown below. Select the template **MycAssay Pneumocystis SDS1_4v3_1.sdt** from the CD-ROM via **Browse...**

2.5 Give the run an appropriate Plate Name. An example is shown below:



2.6 Click **Finish**. A new document will open containing the PCR parameters and detectors automatically set for this assay. In the **Plate** view of the **Setup** tab, use **Well Inspector** (select a well and press Ctrl+1 or right-click with the mouse) to name the wells according to the positions of the samples in 1.14. For example:



2.7 When all the wells are named appropriately, save the run, keeping the **Plate Name** as the file name

2.8 Start the run in the **Instrument** tab by clicking on the **Start** button.
To determine how long the run will take to complete, a countdown is shown next to the **Start** button.

3. Data Analysis and Interpretation

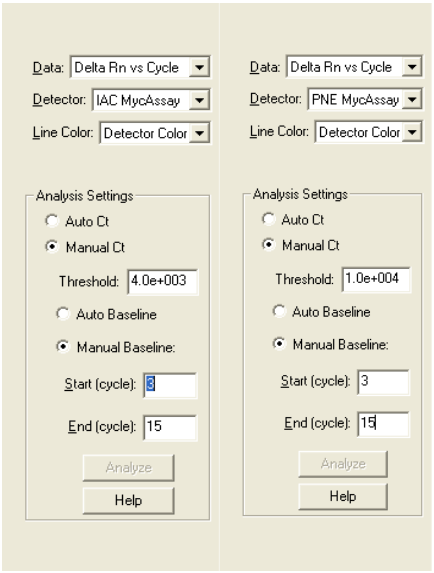
3.1 Once the run has finished, click on the green arrow on the top menu bar to update.

3.2 Open **the Amplification Plot** view of the **Result** tab. On the right hand side set the thresholds for each channel as follows
:.

PNE MycAssay = 10000
IAC MycAssay = 4000

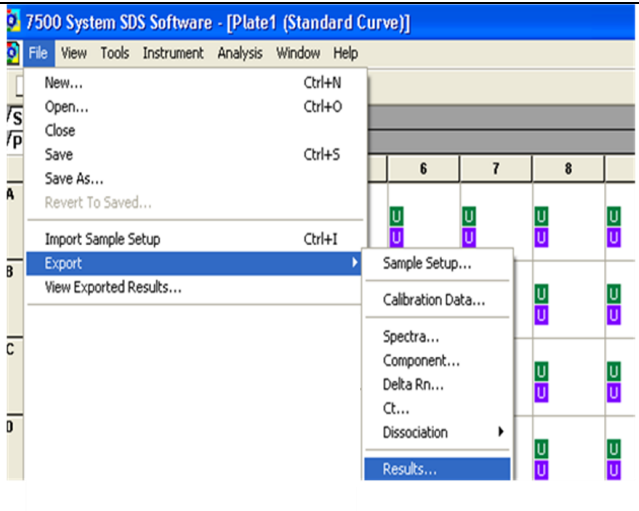
The **Manual Baseline** should remain at 3 - 15 for both detectors.

3.3 Click the **Analyze** button to activate these changes. For example:



3.4 Save the changes.

3.5 Select the wells containing samples and export the Report file **File**>**Export**>**Results...**as shown below:

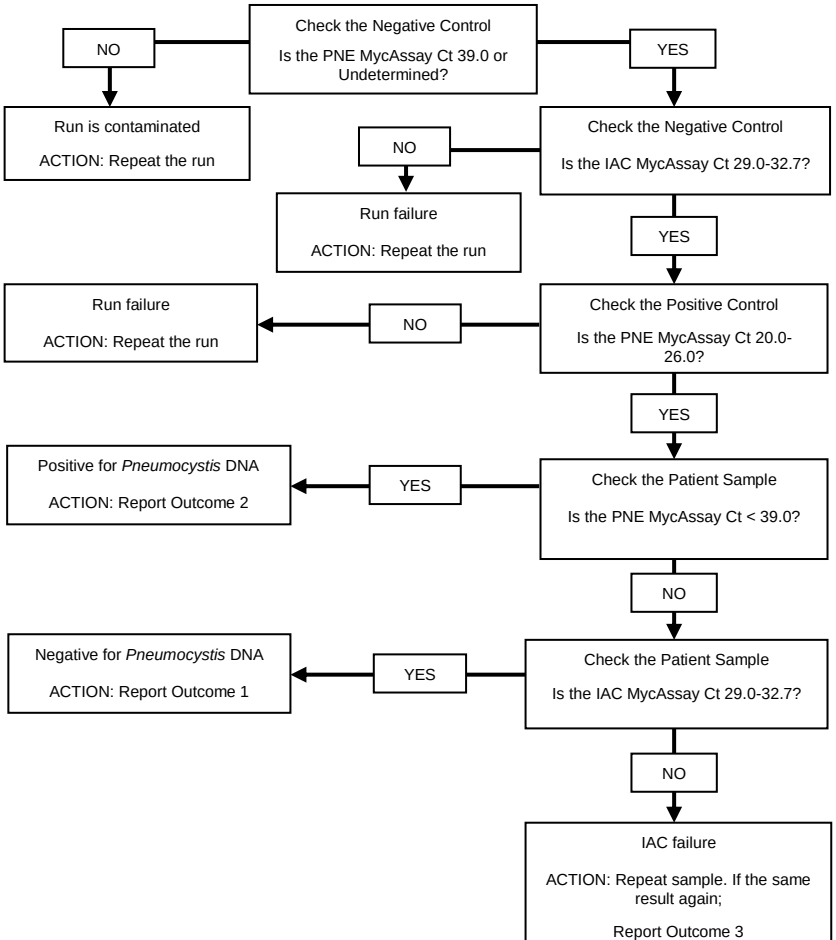


3.6 To avoid confusion, save the file with the same name as used for the run file itself. Remember to save the file to an appropriate location.

3.7 When prompted, activate **Export only selected wells**, and click **OK**.

3.8 Open the saved .csv file with Excel or similar spreadsheet software.

3.9 Analyse each sample, starting with the controls, as shown in the flowchart below (details can also be found in the table shown beneath the flowchart):


Check the Negative Control
Is the PNE MycAssay Ct 39.0 or Undetermined?
NO
YES
Run is contaminated
ACTION: Repeat the run
Check the Negative Control
Is the IAC MycAssay Ct 29.0-32.7?
NO
Run failure
ACTION: Repeat the run
YES
Run failure
ACTION: Repeat the run
NO
Check the Positive Control
Is the PNE MycAssay Ct 20.0-26.0?
YES
Positive for *Pneumocystis* DNA
ACTION: Report Outcome 2
YES
Check the Patient Sample
Is the PNE MycAssay Ct < 39.0?
NO
Negative for *Pneumocystis* DNA
ACTION: Report Outcome 1
YES
Check the Patient Sample
Is the IAC MycAssay Ct 29.0-32.7?
NO
IAC failure
ACTION: Repeat sample. If the same result again;
Report Outcome 3

Sample	PNE MycAssay Ct	IAC MycAssay Ct	Interpretation	Further Action
Negative Control	39.0 or Undetermined	Within 29.0-32.7	Negative Control acceptable	Patient results are valid
Negative Control	39.0 or Undetermined	<29.0 or >32.7	Failure in Negative Control	Repeat entire run
Negative Control	<39.0	Within 29.0-32.7	Contamination	Repeat entire run
Positive Control	Within 20.0-26.0	N/A	Positive Control acceptable	Patient results are valid
Positive Control	<20.0 or >26.0	N/A	Failure in Positive Control	Repeat entire run
Patient	39.0 or Undetermined	Within 29.0-32.7	Negative for *Pneumocystis*	Report result: Outcome 1*
Patient	<39.0	N/A	Positive for *Pneumocystis*	Report result: Outcome 2*
Patient	39.0 or Undetermined	<29.0 or >32.7	IAC failure in sample	Repeat sample: Outcome 3*

*See **Clinical Reporting** for Outcome 1, 2 or 3

4. Troubleshooting

4.1 The Negative Control has generated a positive signal in the FAM channel:

- Contamination occurred during the set up. Results from the entire run cannot be relied upon as accurate.

→ Repeat the entire run taking great care when adding the templates, in particular, the Positive Control (Tube 4), to ensure that cross-contamination does not occur.

→ Make sure that the work area and instruments are properly decontaminated before and after use.

- The Negative Control was incorrectly positioned in the instrument.

→ Take care that all the reactions are correctly annotated within the software, and that the tube strips are placed into the machine in the correct orientation.

- Non-recommended tubes or plates were used.

→ Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

4.2 The Negative Control IAC Ct value is not within the acceptable range:

- The PCR has been inhibited.

→ Ensure that the work area and instruments are thoroughly dry after the use of decontaminating agents prior to PCR set up.

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with unexpired kit if necessary.

- Either Tube 1 or 2 reagent was not added to the PCR reaction, or double the amount of Tube 2 was added.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing higher or lower levels of liquid in one reaction tube compared to others.

- Non-recommended tubes or plates were used.

→ Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

4.3 The Positive Control is negative/out of range:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- An error occurred during setup and the Positive Control template (Tube 4) was placed in the wrong reaction tube.

→ Repeat the run, taking great care during the set-up stage. Such errors can be detected by seeing a higher level of liquid in one reaction, and a lower level in another, compared to normal.

- Either Tube 1 or 2 reagent was not added to the reaction.

→ Repeat the run taking care in the set-up stage. Such errors can be detected by seeing lower levels of liquid in this reaction compared to others.

- The Positive Control was incorrectly positioned in the instrument.

→ Take care that all the reactions are correctly annotated within the software, and that the tube strips are placed into the machine in the correct orientation.

- Non-recommended tubes or plates were used.

→ Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

4.4 Patient sample(s) give Outcome 3 - "Invalid":

- It is likely that the extracted clinical sample(s) contain PCR inhibitors.

→ We recommend that DNA from clinical samples is extracted using the MycXtra™ Fungal DNA Extraction kit.

4.5 There are no results for any channel with any samples or controls:

- The storage conditions of the kit did not comply with the instructions in the Storage section of this IFU, or the kit has expired.

→ Please check correct storage conditions of the kit have been followed. Check the expiry date of the reagents (see the kit box / pouch label) and repeat with an unexpired kit if necessary.

- The equipment used is not functioning optimally.

→ Please check that your Real-Time PCR instrument has an up-to-date service history and has been fully calibrated as described in its Installation and Maintenance Guide.

- An incorrect protocol file was used during the software set up.

→ Please refer to Section 2 and choose the correct Protocol file, as specified for each software type/version, from the Myconostica Protocol CD-ROM. Only the file appropriate to the software can be loaded. Repeat the run using the correct protocol file.

- Non-recommended tubes or plates were used.

→ Thresholds are only valid when using the recommended MicroAmp® tubes and caps.

If you have further questions, or you experience any problems, please contact Technical Support (productsupport@lab21.com)

Performance Characteristics and Limitations

All analytical and clinical performance characteristics and claims for the MycAssay™ Pneumocystis assay were originally determined on the Cepheid SmartCycler. In order to validate the transfer of the assay to the AB7500 Real Time PCR platform, and to demonstrate equivalence between the 2 platforms, certain analytical studies were repeated on the AB7500 platform. The results of those studies are documented below. Studies which were performed on the Cepheid SmartCycler® are indicated.

Analytical Sensitivity

Using the protocol described above, and a recombinant *Pneumocystis* DNA molecule generated at Myconostica, the Limit of Detection (LoD) for *Pneumocystis* was determined to be < 30 copies. This value was determined using a recombinant DNA plasmid harbouring the target sequence. The *Pneumocystis* target sequence is mitochondrial, therefore, there will be numerous copies per cell, but it is not known how many.

Analytical Selectivity

Analytical selectivity was tested using DNA extracted from a variety of different fungal and non-fungal species. The following species did not report out a positive result;
Alternaria alternata, Aspergillus flavus, A. fumigatus, A. niger, A. terreus, Blastomyces capitatus, Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, Cladosporium spp., Cryptococcus neoformans, Doratomyces microsporus, Fusarium solani, Histoplasma capsulatum, Rhizomucor pusillus, Rhodotonila rubra, Saccharomyces cerevisiae, Scedosporium apiospermum, S. prolificans, Sporothrix schenkii, Trichosporon capitatum The following bacterial species did not report a positive result; *Bordetella pertussis, Corynebacterium diphtheriae, Escherichia coli, Haemophilus influenzae, Lactobacillus plantarum, Legionella pneumophila, Moraxella catarrhalis, Mycoplasma pneumoniae, Neisseria meningitidis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae, S. pyogenes, S. salivarius*.

Human genomic DNA does not report a positive result with this assay.

Interfering Substances (contraindications for use)

(The following data were generated with the MycAssay™ Pneumocystis kit during validation studies using the Cepheid SmartCycler®. Validation was performed to demonstrate equivalence of performance between platforms.)

The following compounds were tested at clinically relevant concentrations, and found not to inhibit the assay; acteylcysteine, amphotericin, beclometasone dipropionate, budesonide, colistimethate sodium, fluticasone propionate, formoterol fumarate

dihydrate, ipratropium bromide, lidocaine, mannitol, salbutamol sulphate, salmeterol, septrin (trimethoprim-sulphamethoxazole), sodium chloride, sodium cromoglicate, terbutaline and tobramycin.

Performance Evaluation

(The following data were generated with the MycAssay™ Pneumocystis kit during validation studies using the Cepheid SmartCycler®. Validation was performed to demonstrate equivalence of performance between platforms.)

The clinical cut-off at a Ct of 39.0 was established following analysis of a set of clinical samples sourced from different patient populations.

Clinical samples collected by bronchoaleveolar lavage (BAL) that had been obtained at 2 hospitals, extracted using the MycXtra® kit, and stored, were used to evaluate the performance of the MycAssay™ Pneumocystis kit. Comparisons were made of the PCR results to immunofluorescent microscopy.

PCR v Microscopy Diagnosis

	Microscopy positive	Microscopy negative		
PCR positive	45	8	0.85	PPV
PCR negative	2	33	0.94	NPV
	0.96	0.80		
	Sensitivity	Specificity		

Table 1: Diagnostic specificity and sensitivity of the MycAssay™ Pneumocystis kit compared to immunofluorescent microscopy.

Table 1 represents data obtained from patients with diagnosed HIV, patients not infected with HIV and patients with undetermined HIV status. Patients with *Pneumocystis* pneumonia have highly variable amounts of organism detectable; the lower the Ct value the higher the likelihood of disease. Patients with HIV and *Pneumocystis* pneumonia tend to have higher numbers of organisms detectable than patients who are not infected with the virus, but the overlap is considerable. The scatter plot in Figure 1 below demonstrates this overlap. For completion, as the data-set in Table 1 included patients whose HIV status was unknown, the scatter plot for this group is included in Figure 1 (column 3):

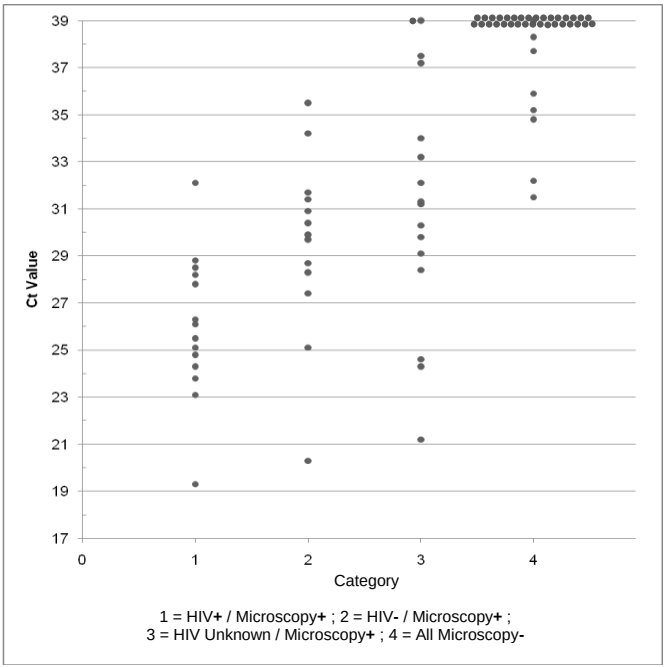


Figure 1: Scatter plot of Ct values obtained from DNA extracted from patient respiratory samples. Four groups are described.

Clinical Reporting

The MycAssay™ Pneumocystis kit is intended as an aid to diagnosis of Pneumocystis pneumonia. The results need to be taken in context of the clinical condition of the patient and other diagnostic tests results.

The following are recommended reports, each depending on the assay result interpretation.

Outcome No 1
"*Pneumocystis jirovecii* not detected."

Outcome No 2
"*Pneumocystis jirovecii* detected. Positive result. State Ct value"

Outcome No 3
"Test failed; inhibitors or other unknown substance present."

The lower the Ct value the higher the probability of disease. Ct values close to the cut-off of 39.0 are more likely to represent colonisation than infection, but some patients may have disease with very little *P. jirovecii* present, representing a poor specimen, prior treatment or the nature of fungal load in that particular patient.

Limitations of Procedure

- The principal limitation of this procedure relates to the quality of the primary sample:
  - If the sample is very small or not collected from the affected area of lung, the test will be less sensitive and may be falsely negative.
  - BAL samples should be centrifuged prior to DNA extraction from the pellet.
  - Data also demonstrated that a reduction in the volume of supernatant used in the extraction process, achieved by the centrifugation step, decreases the proportion of inhibitors entering the system.

- While the MycXtra™ Fungal DNA extraction procedure is designed to remove PCR inhibitors, not all drugs or patient populations have been evaluated.
- The procedure has not been fully assessed with sputa nor has it been assessed with induced saline samples or on samples from children.
- False positive results may arise from external contamination of the original sample or test. Such contamination could arise from *P. jirovecii* contaminated air, poor experimental technique with respect to the positive control or external (especially pipette) contamination with *P. jirovecii* DNA.
- As a true positive result may be obtained from patients who are transiently or persistently colonized by *P. jirovecii*; clinical judgment is required in interpretation of the test result.

LICENSING

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